# Chromatography of Polymers

## Characterization by SEC and FFF

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Chapter 22

# Structural Analysis of Aggregated Polysaccharides by High-Performance Size-Exclusion Chromatography–Viscometry

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Three high performance size exclusion columns placed in series were calibrated in terms of radii of gyration ($R_g$) and hydrodynamic volume (intrinsic viscosity ([IV]) x molecular weight (M)) with a series of pullulan and dextran standards ranging in M from 853,000 to 10,000. Online detection was by differential refractive index (DRI) and viscometry (DP). Two forms of universal calibration were employed to obtain $R_g$, [IV], and M for pectin, a class of complex plant cell-wall polysaccharides. For pectins extracted from a large number of sources, a Mark-Houwink plot (log [IV] vs. log M) gave a correlation coefficient of 0.2; whereas, a plot of log [IV] against log $R_g$ gave a correlation coefficient of 0.9. These results in addition to those from the analysis of several pectins from peach fruit indicated that pectins were aggregated and highly asymmetric in shape. The scaling law exponents for pectins were effected by both shape and state of aggregation, rather than shape alone.

The tendency to aggregate significantly complicates the structural analysis of many polysaccharides (1). Pectin is an example of aggregated polysaccharides (2,3), consisting of a group of closely related anionic polysaccharides found in the cell walls of all higher plants (4). Recently, we have shown that pectin from a variety of sources is an associated colloid comprised of five macromolecular-sized species when analysed by HPSEC (5,6). By employing HPSEC/viscometry with curve fitting of the chromatograms and two forms of universal calibration (7), we obtained the root mean square (rms) radii of gyration ($R_g$), intrinsic

viscosities ([IV]), and molecular weights (M) of the five components and their global averages. Here, we examine the effect of aggregation on the inter-relationships between [IV], $R_g$, and M.

## Experimental

**Materials.** Pectins, referred to as "by-product" pectins, were extracted from beet pulp, the peels of mangoes, oranges, mandarin oranges, grapefruits, pomegranates, and artichokes, the skin of garlic and peas, carrot and colocasia wastes, and garlic foliage. Typically the pectin source was extracted for 1 h at 90°C with 0.5 % w/v ammonium oxalate solution, precipitated with acidified alcohol, and dried (8). Then, 1g of the dried pectin was dissolved in 60 mL of 0.01 M sodium phosphate buffer containing 0.01M EDTA (pH 6.05), stirred overnight at 4°C, dialyzed against 4 changes of water over 48 h, centrifuged at 32,000g for 1 h at 5°C to remove trace insolubles, and lyophilized.

Peach pectins were obtained from the mesocarp of hard, melting flesh (MF) "Redskin" peaches and from non-melting flesh (NMF) "Suncling" peaches. Chelate soluble (CSP) and mildly alkaline soluble pectin (ASP) were extracted sequentially from isolated, washed cell walls according to a procedure described by Gross (9).

**Chromatography.** High performance size exclusion was performed as reported earlier (10). Pectin, dissolved in 0.05 M $NaNO_3$ or NaCl, was passed through a 0.4 $\mu$M Nucleopore filter and equilibrated overnight at 35°C in capped bottles prior to chromatography. The mobile phase was either 0.05 $NaNO_3$ or NaCl in HPLC grade water. Solvent was degassed prior to connecting to the system and inline with a model ERC 3120 degasser, Erma Optical Co., Tokyo. The solvent delivery system was a model 334, Beckman Instr., Palo Alto, CA. The nominal flow rate was 0.5 mL/min. The pumping system was fitted with a Beckman pulse filter and two model M45 pulse dampners, Waters Assoc, Millford, MA, mounted on a plate and separated by 15 ft of coiled capillary tubing (i.d., 0.01 inches). Sample injection was with a Beckman model 210 valve. The injected sample volume was 100 $\mu$L. Three columns were employed in series, a Micro-Bondagel E-High A, E-1000, Waters Assoc.(300x3.9 mm) and a Synchropak GPC-100 (250x4.6 mm) Synchrom, Inc., Linden, IN. The viscosity detector (differential pressure detector, DP) was a model 100 differential viscometer, Viscotek Corp., Porter, TX or an inhouse single coil model described in reference 10. When viscosity detection was with the model 100, injected sample concentrations ranged from 0.53 to 0.57 mg/mL (i.e., peach pectins) whereas viscosity detection with the inhouse model required sample concentration in the range 2.5 to 2.7 mg/mL ("by-product" pectins). Differential refractive index (DRI) was measured with a model ERC 7810 monitor, Erma Optical Co., Tokyo. Chromatography columns were thermostated in a temperature controlled water bath at 35 $\pm$ 0.003°C and the cells of the refractive index and viscosity monitors were thermostated also at 35°C. Data collection and flow rate measurement have been described previously (11).

**Curve fitting.** The partially resolved, overlapped components of the DRI and the DP detector chromatograms were determined with the aid of ABACUS, version D.2, a nonlinear least-squares curve fitting program. The dead volume between the DRI and DP detectors was measured as 125 ± 1 μL by matching the front end of chromatograms from a narrow P-50 pullulan standard normalized for area from the respective detectors. For fitting of DP traces, values of component peak position, quarter width at half height (sigma) and number of Gaussian peak components were obtained from DRI traces as already described (5). Since the two detectors only differed in their sensitivity of response towards pectin, only peak heights were iterated until the sum of the squares of the point by point residuals between the calculated curve reconstructed from the components and the experimental trace were minimized to convergence.

**Column Calibration.** As previously described congruent calibration curves were obtained by plotting log $R_g$ or log [IV]M against column partition coefficient,($K_{av}$), for a series of narrow molecular weight distribution (MWD) pullulan and broad MWD dextran standards (i.e., dextrans with polydispersities ranging from 1.39 to 2.91) (5,7). These calibration curves were used to obtain $R_g$ and M for unknown pectin samples by the "universal calibration" procedure. According to this procedure, pectins will co-elute with dextrans and pullulans which have identical values of either $R_g$ or product of [IV] and M. Values of M or $R_g$ for pectins were calculated by transforming partition coefficients along the pectin refractive index response to $R_g$ or to the product [IV]xM. Transformations were obtained from the dextran-pullulan calibration curves (11). To obtain M as a function of $K_{av}$, the product of [IV]M as a function of $K_{av}$ was divided by [IV] which also was obtained as a function of $K_{av}$ by the online viscosity detector. In cases where component analysis was possible, the molecular weight or radius of gyration for the component was obtained from the peak maximum of the component. The weight fraction of the component was obtained from the component area under the refractive index trace. Weight average properties were obtained by summing over the components as described previously (7). In cases where component analysis was not possible, continuum calculations were carried out. In these cases, corrections for bandspreading were made by the GPCV2 procedure (12).

## Results and Discussion

For a macromolecule dissolved in a good solvent at constant temperature, [IV], $R_g$, and M are interrelated through equation 1, the modified Einstein equation (13).

$$[IV] = A(R_g)^3 / M \qquad (1)$$

In the case of a single, linear polymer chain, an increase in the degree of polymerization will result in increases in intrinsic viscosity ([IV]), radius of gyration ($R_g$), and molecular weight (M), in such a manner as to maintain a constant value of A, the proportionality constant in equation 1. In the case of

pectin, a highly asymmetric, aggregated, polyelectrolyte (2), the question arises as to whether some circumstances might exist in which [IV] and M, are not dependent on $R_g$. A Mark-Houwink plot was constructed for the 12 "by-product" pectins by plotting log [IV] against log $M_w$ (weight average molecular weight) (Figure 1). $M_w$ was calculated from five curve-fitted components of the HPSEC chromatograms as described previously (7). The correlation coefficient for this data was 0.2 whereas a plot of log [IV] against log $R_{gw}$ (weight average radius of gyration) (Figure 2) gave a correlation coefficient of 0.9. The Mark-Houwink scaling law exponent was 0.38 which is the value expected for a macromolecule more compact than a random coil in an ideal solvent (14). A value of 0.89 was found for the scaling law exponent, x, relating $R_g$ and $M_w$, which is obtained from equation 2 (10).

$$[IV] = C(R_g)^{3 - (1/x)} \qquad (2)$$

The value of x is close to the expected value for a rigid rod (15).

The finding that [IV] was more highly correlated with $R_g$ than it was with M was tested further by measuring [IV], $R_g$, and M for pectins from two solubility fractions in each of two varieties of peaches. In the case of the chelate soluble pectin (CSP) fractions unlike the alkaline soluble pectin (ASP) fractions, differential refractive index (DRI) and differential pressure (DP) chromatograms could not be fitted with the same set of components (cf. ASP and CSP chromatograms from (non melting flesh) peaches in Figures 3 and 4). Thus the four pectins were compared with weight average global parameters rather than parameters for the components. As indicated by the data in Table I, for the two varieties, $R_g$ and [IV] are substantially higher in the CSP fraction than in the ASP fraction whereas M differs much less between the two fractions and does not appear to be highly correlated with [IV].

Previously (5), we have shown that the larger pectin components can be dissociated into smaller ones by dialysing against 0.05 M NaCl. In the course of three separate but similar extractions of ASP from melting flesh peaches, the largest component of sample 3 appears to have undergone dissociation during extraction. The results from these experiments are found in Table II. For the largest component in all three extractions, $R_g$ was about the same, 42.2 $\pm$ 1.9 nm whereas there were appreciable differences in [IV] and M for samples 1 and 3, ranging from 5.7 $\pm$ 0.3 dL/g and 316 $\pm$ 29 x $10^3$ to 3.62 $\pm$ 0.01 dL/g and 458 $\pm$ 13. In accordance with equation 1, at constant $R_g$, [IV] was inversely related with M. Such behavior would be possible under circumstances in which $R_g$ and M were independent variables. One example would be two highly asymmetric molecules with identical contour lengths but differing in thickness, e.g., two aggregated rods which differed in degree of aggregation but not in length. For rod-like molecules, $R_g$ and M are dependent variables when length changes but independent variables when only thickness changes. Furthermore increases in molecular weight which are only related to increases in thickness decrease viscosity whereas increases in molecular weight which are only related to increases in length increase viscosity.

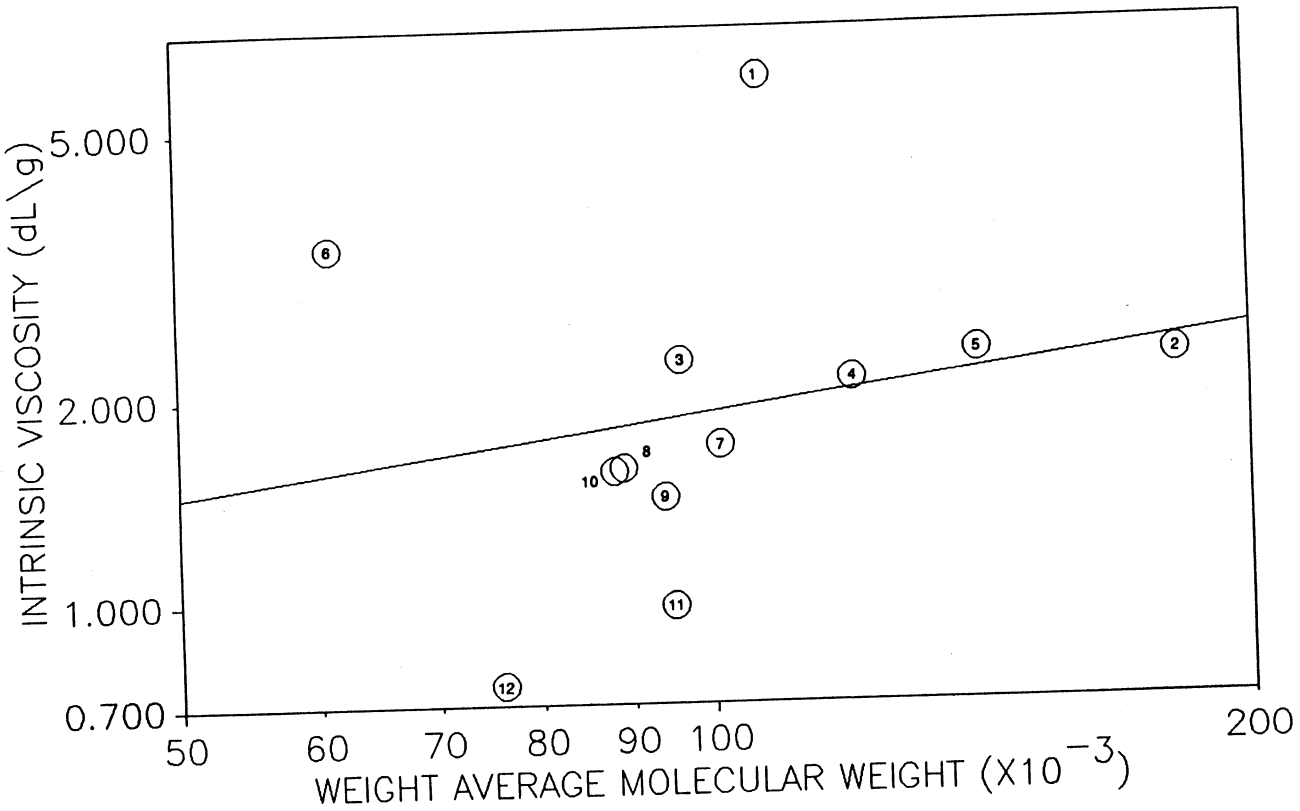


Figure 1. Mark-Houwink Plot of "By-product" Pectins. 1. Pomegranate Peels, 2. Carrot Waste, 3. Beet Pulp, 4. Orange Peel, 5. Artichoke Peel, 6. Colocasia Waste, 7. Mandarin Orange Peel, 8. Mango Peel, 9. Grapefruit Peel, 10. Pea Skin, 11. Garlic Skin, 12. Garlic Foliage.

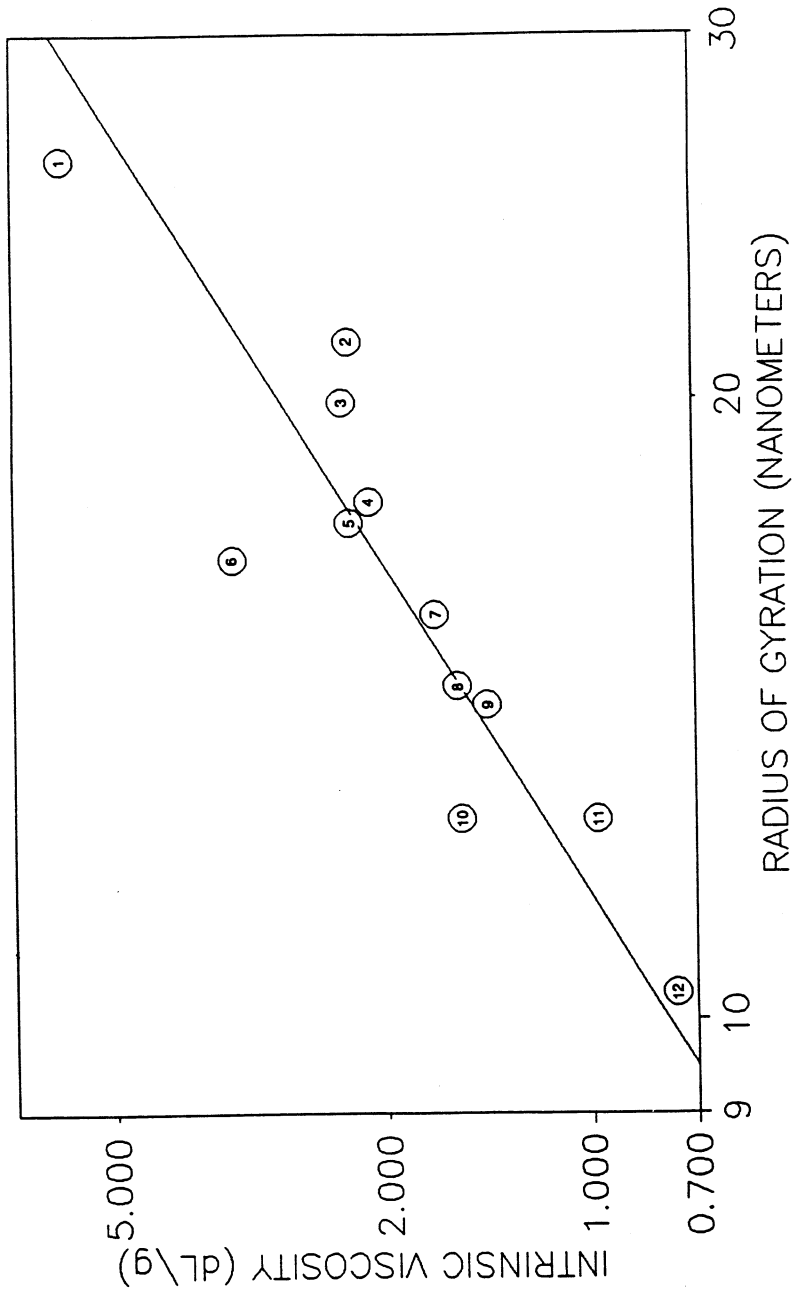


Figure 2. Double Logarithmic Plot of Intrinsic Viscosity Against Radius of Gyration of "B Product" Pectins. Key as in Figure 1.

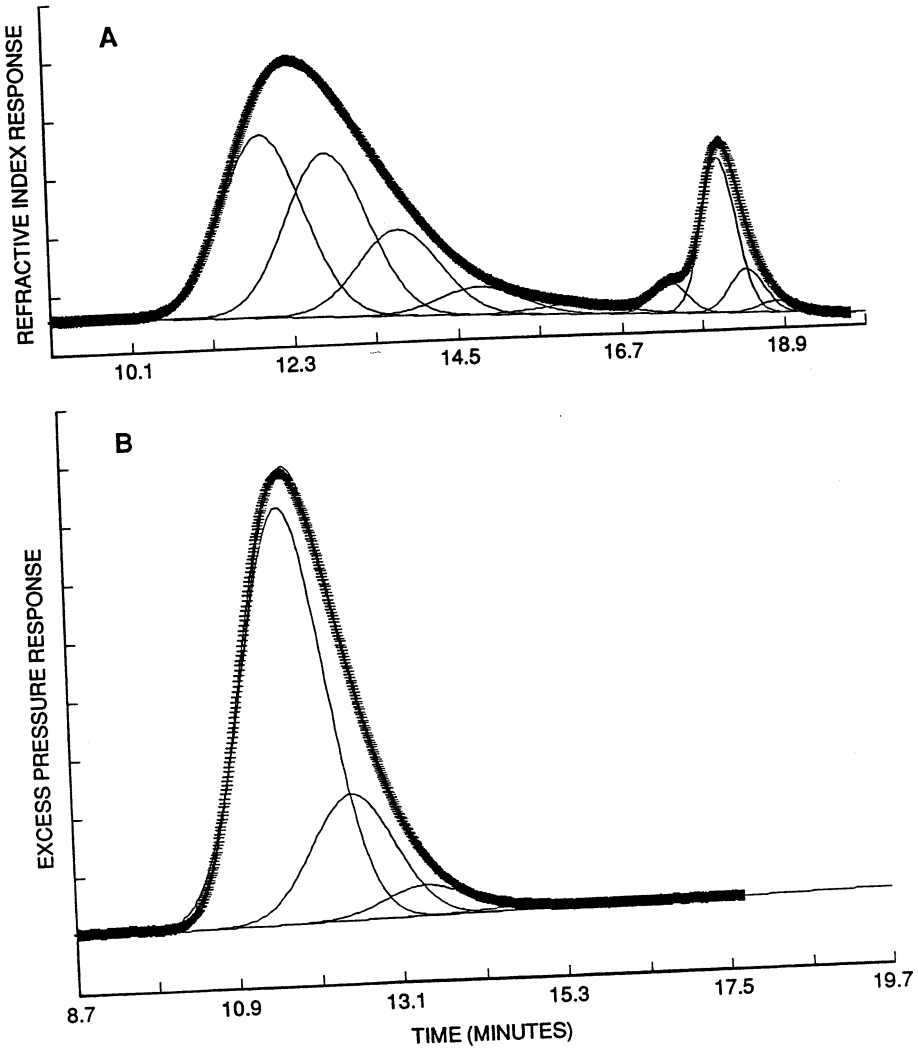


Figure 3. Chromatograms of Alkaline Soluble Pectin Extracted from Cell Walls of "Non Melting Flesh Peaches". Mobile phase, 0.05 M $NaNO_3$; nominal flow rate, 0.5 mL/min.; injection volume, 100 $\mu$L; Injected concentration 0.55 mg/mL. Thick line, experimental; thin line, calculated detection. Macromolecular components referred to in text are numbered 1-5, left to right. (A) Detector, differential refractive index; (B) detector, differential pressure (differential viscosity).

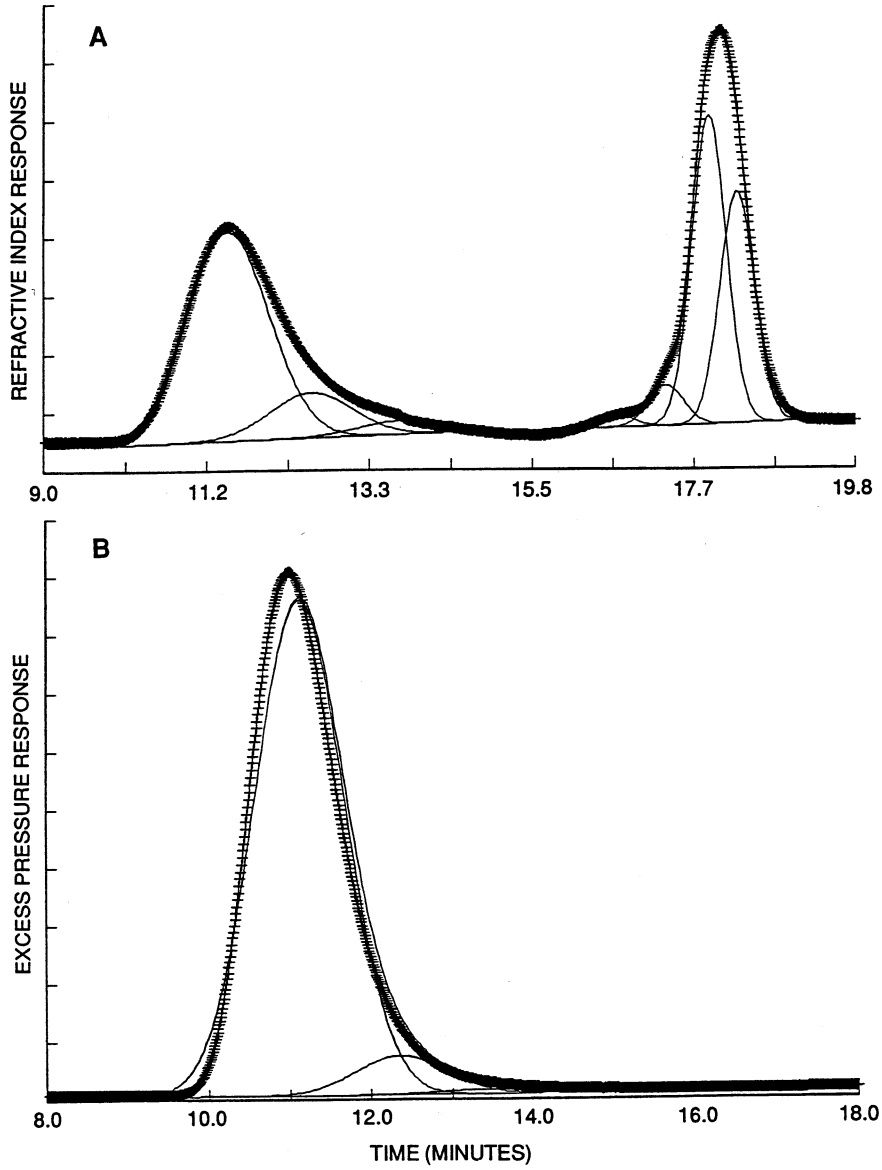


Figure 4. Chromatograms of Chelate Soluble Pectin Extracted from Cell Walls of "Non Melting Flesh Peaches". Mobile phase, 0.05 M $NaNO_3$; nominal flow rate, 0.5 mL/min.; injection volume, 100 $\mu$L; Injected concentration 0.53 mg/mL. Thick line, experimental; thin line, calculated detection. Macromolecular components referred to in text are numbered 1-5, left to right. (A) Detector, differential refractive index; (B) detector, differential pressure (differential viscosity).

**Table I. Comparison of Weight Average Properties of Pectins from Two Varieties of Peaches**

| Fraction | CSP | | | ASP | | |
|---|---|---|---|---|---|---|
| Variety | Rg[1] | [IV][2] | Mx$10^{-3}$ | Rg | [IV] | Mx$10^{-3}$ |
| NMF[3,4] | 49.4±0.9 | 11.8±0.3 | 206± 4 | 25.5±2.0 | 3.3±0.5 | 159±12 |
| MF[5] | 40.6±0.9 | 12.2±0.3 | 125±23 | 27.0±2.0 | 3.0±0.5 | 204±16 |

[1]Nanometers [2]dL/g [3]non melting flesh [4]±S.D. of 3 measurements [5]melting flesh

**Table II. Properties for Components from ASP "Melting Flesh" Pectin**

| Component | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Weight Fract.[1] | | | | | |
| Sample 1 | 0.408±0.00 | 0.356±0.00 | 0.184±0.00 | 0.052±0.00 | ---------- |
| Sample 2 | 0.365±0.00 | 0.350±0.00 | 0.200±0.00 | 0.058±0.00 | 0.027±0.002 |
| Sample 3 | 0.346±0.00 | 0.347±0.00 | 0.227±0.00 | 0.066±0.00 | 0.015±0.002 |
| $R_g$ (nm) | | | | | |
| Sample 1 | 43.4±1.3 | 22.3±0.5 | 12.7±0.3 | 7.8±0.2 | ---------- |
| Sample 2 | 40.4±0.3 | 20.3±0.1 | 11.3±0.1 | 6.5±0.1 | 2.9±0.1 |
| Sample 3 | 41.9±0.5 | 20.2±0.3 | 11.4±0.2 | 6.9±0.3 | 3.3±0.2 |
| [IV] (dL/g) | | | | | |
| Sample 1 | 5.7±0.3 | 2.31±0.05 | 1.08±0.09 | 0.43±0.11 | ---------- |
| Sample 2 | 5.2±0.1 | 2.32±0.02 | 0.85±0.03 | 0.55±0.01 | 0.55±0.07 |
| Sample 3 | 3.62±0.01 | 2.43±0.03 | 0.63±0.03 | 0.73±0.04 | 0.95±0.18 |
| % Sp $V^2$ | | | | | |
| Sample 1 | 69.0±1.3 | 24.5±1.4 | 5.9±0.3 | 0.7±0.2 | ---------- |
| Sample 2 | 64.8±0.2 | 27.7±0.2 | 5.8±0.2 | 1.1±0.1 | 0.5±0.1 |
| Sample 3 | 54.4±0.4 | 36.6±0.1 | 6.3±0.3 | 2.1±0.1 | 0.6±0.2 |
| M x $10^{-3}$ | | | | | |
| Sample 1 | 316±29 | 155±11 | 71±5 | 45±17 | ---------- |
| Sample 2 | 291± 5 | 122± 2 | 65±2 | 19± 1 | 0.18±0.02 |
| Sample 3 | 458±13 | 111± 2 | 90±6 | 17± 3 | 0.15±0.06 |

[1]Sample 1 average ± S.D. of 5 measurements; samples 2 and 3 average ± S.D. of 3 measurements. [2]Percentage specific viscosity.

Table III contains [IV], $R_g$ and molecular weight values for components of two groups of "by-product" pectin. Each of these groups was chosen because their components had $R_g$ values that were closely similar. Component 1 of beet, orange, and carrot pectin; and components 1 and 2 of pea skin, grapefruit and garlic skin pectin had [IV]'s which were different. As with the peach components, there is an inverse relationship between [IV] and molecular weight for these components.

## Conclusions

These results are consistent with the hypothesis that pectin is comprised of aggregated rods, aggregated segmented rods or a combination of both. The low correlation of log [IV] and log M and the relatively higher correlation of log [IV] and log $R_g$ is consistent with the finding that pectins of comparable $R_g$ have [IV]'s and M's which are inversely related, if they are aggregated to different extents. Furthermore, such a situation is consistent with the modified Einstein Law relating [IV], M, and $R_g$.

In this work, we have produced evidence that asymmetric molecules which are aggregated to different extents but have identical $R_g$ values will co-elute on a size exclusion column in spite of differing [IV] and M values. An important consequence of this finding is that for these kinds of macromolecules, e.g., pectins, universal calibration rather than calibration of the column by pectins of "known" molecular weight could be a better procedure for determining molecular weights by HPSEC.

Polysaccharides are ubiquitously distributed throughout the world of plants, animals and microorganisms (16). They are important industrially and in biological processes. Although they are involved in a variety of roles, many details remain to be learned at the molecular level concerning structural-functional relationships between polysaccharides and the complex systems in which they exist. As an example, in the case of pectin, the work in this report and several others (5-7,10,17) indicates the existence of pectin quaternary structure. We believe that a better knowledge of pectins' quaternary structure and under what conditions it changes is extremely important in understanding the mechanisms by which pectin functions as a dietary fiber which lowers blood cholesterol and reduces glucose intolerance in diabetics, contributes to the texture of fruits, vegetables, and their processed products, acts as a chemical messenger to defend plants against attack by pathogens and induces metabolic processes important in plant growth, development and senescence (18). A better understanding of these mechanisms would supply information which could aid in delaying heart failure, reducing the incidence of certain cancers through proper nutrition; and aid in the development of more disease-resistant plants whose edible products would taste better, would be less susceptible to post harvest deterioration, and would be more readily processed.

Table III. Properties of Components from "By-Product" Pectins

| Component | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| $R_g$[1] (nm) | 39.7±1.0 | 20.7±1.0 | 10.9±0.7 | 5.4±0.4 | 2.6±0.2 |
| [IV] (dL/g) | | | | | |
| Beet | 4.4±0.1 | 2.7±0.1 | 0.94±0.04 | 0.90±0.23 | 1.68±0.24 |
| Orange | 4.0±0.1 | 2.9±0.1 | 0.94±0.04 | 0.89±0.16 | 0.82±0.09 |
| Carrot | 3.6±0.1 | 2.7±0.1 | 1.07±0.05 | 0.99±0.25 | 1.06±0.49 |
| M x $10^{-3}$ | | | | | |
| Beet | 383±33 | 120±12 | 60±9 | 9.7±3.8 | 0.5 ±0.1 |
| Orange | 404± 8 | 95± 6 | 48±5 | 7.0±2.3 | 0.8 ±0.1 |
| Carrot | 475±21 | 125±11 | 42±4 | 6.8±2.3 | 1.1 ±0.5 |
| % Sp V | | | | | |
| Beet | 40.1±1.1 | 41.3±0.4 | 13.0±1.0 | 4.2±0.4 | 1.4±0.3 |
| Orange | 33.5±1.6 | 44.7±0.6 | 16.2±0.5 | 4.5±0.7 | 1.1±0.1 |
| Carrot | 43.5±1.7 | 37.6±1.2 | 13.8±1.4 | 3.9±0.5 | 1.3±0.7 |
| $R_g$[1] (nm) | 34.0±0.8 | 17.6±0.7 | 9.7±0.4 | 5.5±0.2 | 2.8±0.2 |
| [IV] (dL/g) | | | | | |
| Pea Skin | 4.3 ±0.1 | 2.0±0.1 | 0.67±0.01 | 0.38±0.08 | 0.22±0.12 |
| Grapefruit | 3.3 ±0.2 | 2.3±0.1 | 0.78±0.06 | 0.36±0.14 | 0.74±0.36 |
| Garlic Skin | 2.5 ±0.2 | 1.7±0.1 | 0.65±0.03 | 0.27±0.03 | 0.38±0.42 |
| M x $10^{-3}$ | | | | | |
| Pea Skin | 237± 5 | 103± 5 | 51±8 | 13±5 | 4.0 ±2 |
| Grapefruit | 362±33 | 96± 7 | 54±2 | 23±9 | 1.4 ±0.6 |
| Garlic Skin | 444±22 | 111±10 | 52±2 | 26±3 | 6.4 ±4.7 |
| % Sp V | | | | | |
| Pea Skin | 37.5±0.9 | 43.1±0.3 | 16.1±0.9 | 3.1±0.1 | 0.5±0.4 |
| Grapefruit | 26.9±2.6 | 43.4±1.4 | 24.3±2.8 | 4.0±1.6 | 1.4±0.9 |
| Garlic Skin | 23.5±0.9 | 40.8±0.8 | 28.4±1.2 | 6.2±1.2 | 1.1±1.0 |

[1]Average ± S.D. of 3 pectins x 3 replicates = 9 measurements; [IV], M and % SP V (percentage specific viscosity) are average ± S.D. of 3 measurements.

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